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14. ABSTRACT

The purpose of the research done has been to determine the mechanism for overexpression of JAB1 through transcription factor analysis and FISH as well as the role of JAB1 in resistance. The major findings thus far are 1) The promoter region of JAB1 was analyzed and key transcription factors were identified that may drive JAB1 expression, CEBP alpha and GATA1 2) We have performed FISH on a number of breast cancer cell lines and patient fine Needle aspirations and have seen amplification of the JAB1 locus 3) JAB1 overexpression confers resistance to Herceptin in breast cancer cell lines SKBR3 and BT474, and inhibition of JAB1 increased the efficacy of Herceptin mediated G1 arrest and p27 accumulation. We completed the tasks listed for this time period and are on track as indicated in the SOW.

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Introduction

The purpose of the research done has been to determine the key transcription factors that are driving JAB1 expression, determine if JAB1 copy number is amplified in breast cancer, and to evaluate whether JAB1 overexpression in breast cancer plays a role in resistance to Herceptin treatment and whether inhibition of JAB1 will enhance sensitivity to treatment. In the past year, we have accomplished many of the tasks laid out in the Statement of Work (SOW). Tasks 1A, 1B, 1C were completed. We have begun working on Tasks 2A, 3A, and 3B. The data from these tasks are shown in this annual update.

Body

Task 1 of this proposal focused on characterizing the JAB1 promoter and its transcriptional regulation. We have identified the transcriptional start site and putative transcription factor binding sites of the JAB1 gene. In order to isolate and characterize the jab1 gene and its 5'-flanking region, we began by determining the transcriptional start site by primer extension analysis. It was found to be 91 bp upstream of the ATG translational start codon by primer extension analysis. A TATA box and CCAAT box was found within 77bp of this start site. Putative promoter regions were located using Proscan software and a series of 5' deletion mutants were constructed. PCR products varying from 32 to 2000 bp were subcloned into the pGL3 luciferase vector. The minimal promoter was found to be 472 base pairs upstream of the transcriptional start site. The region between –472 and –344 bp upstream of the ATG was essential for transcription. This region contains GATA and C/EBP consensus sequences (Fig 1).

We have successfully identified the transcription factors that drive JAB1 transcription. We used site directed mutagenesis to mutate the DNA-response elements GATA and C/EBP in the region –472 to -344. These mutants were sub-cloned into the pGL3-basic vector and tested for luciferase activity in MCF7 cells. Mutation of both reduced the promoter activity, but C/EBP resulted in almost no activity of the JAB1 promoter and is likely to be very important for JAB1 transcription. To determine which of the C/EBP and GATA family members were important for JAB1 transcription, we transfected C/EBPα, β, and δ along with GATA 1-6 into MCF7 cells. C/EBPα, C/EBPβ, and GATA1 had the greatest increase in activity. Probes containing either the C/EBP or GATA binding sites were used in electrophoretic mobility shift assays (EMSA) in MCF7 and SKBR3 cells. Addition of the cold, specific probe competed for binding while probes with mutations in the binding sites did not (Fig 2). Specific antibodies for C/EBPα, C/EBPβ, and GATA1 were unable to form supershifting protein:DNA complexes. But all were able to bind to this region by chromatin immunoprecipitation (CHIP) analysis.

We have successfully completed the goals of Task1. We will further identify the pathway that is driving transcription of JAB1 through these transcription factors. When this is completed we will submit this work for publishing.

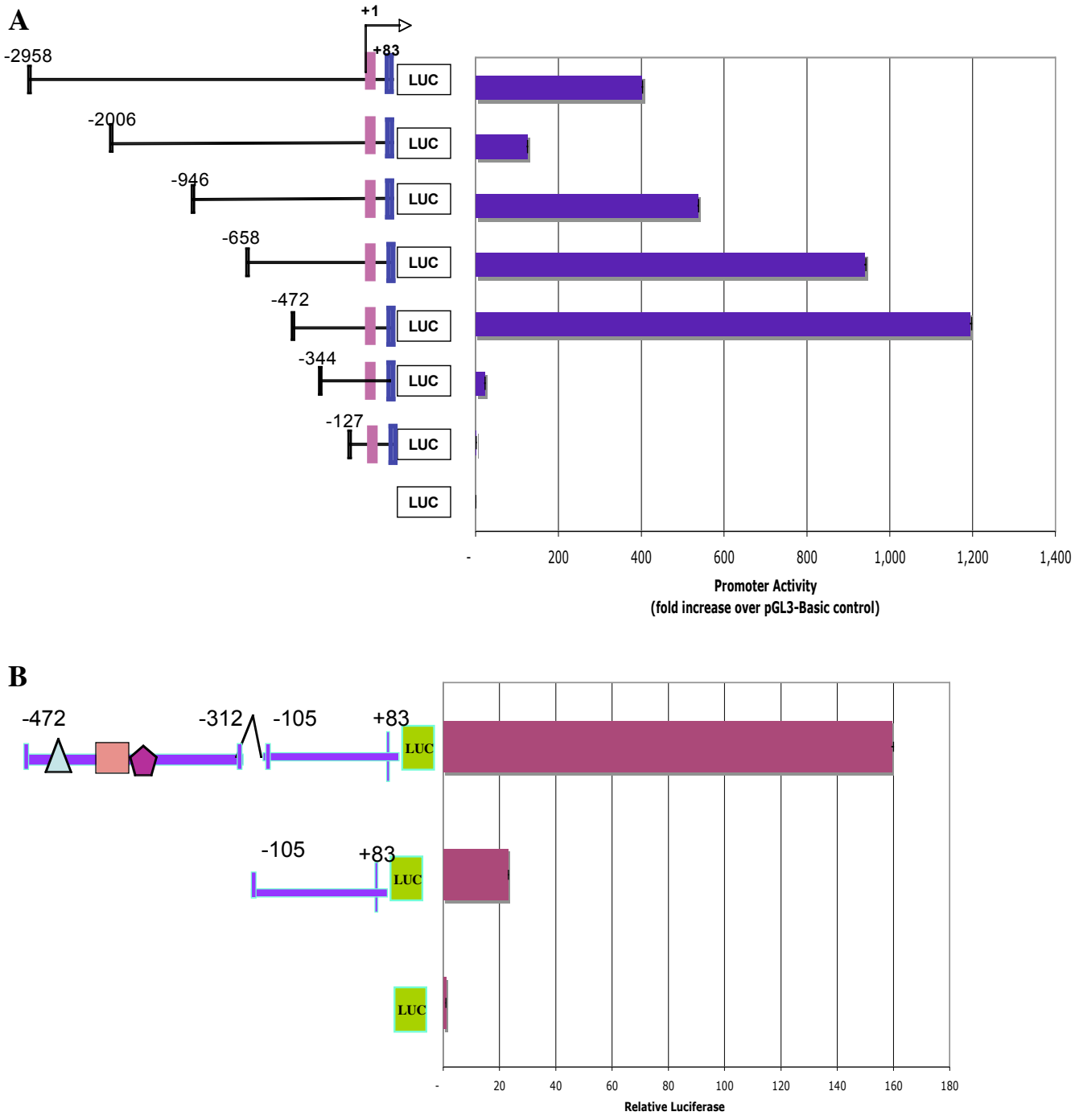
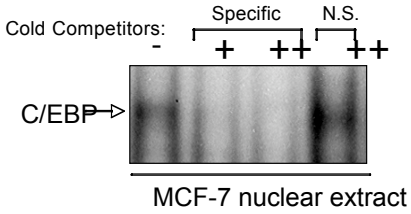


Fig. 1. Identification of the minimal promoter region of JAB1 (**A**) Progressive deletion of the 5'-region in luciferase reporter constructs and (**B**) the minimal promoter region subcloned in front of the TATA and CAAT box were used. The schematic presentation of the luciferase reporter constructs is shown. These plasmids were co-transfected with the Renilla luciferase plasmid pRL-null as a transfection control into MCF7 cells, and luciferase activity was assayed after 36 hours. Relative promoter activity of each construct is shown as fold increase over promoterless luciferase control pGL3-Basic, whose activity is taken as 1, after normalizing to pRL-null activity. Values represent mean +- S.E. of triplicate experiments.

A.

EMSA Probes	Regions	Sequences
C/EBP WT	-462 to -445 bp	CCTTACCTTTTAGTCTTTCAACAAACT
C/EBP Mut.	-462 to -445 bp	CCTTACCTTTTAGTCTTTCctgAAACT
GATA	-444 to -417 bp	AATCATTTATCTCATTTAAGGTACC
GATA Mut	-444 to -417 bp	AATCATTggagTCATTTAAGGTACC



B.

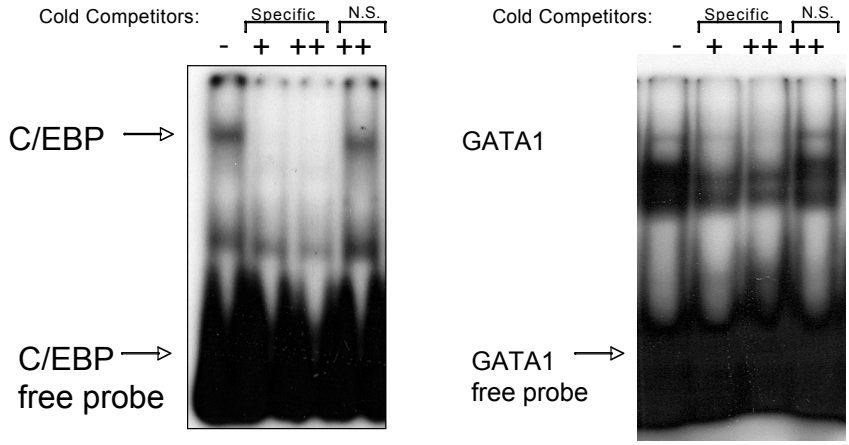


Fig. 2. Characterization of transcription factors interacting with the JAB2 proximal promoter. (**A**), sequence of the probe used in EMSA assays. The probe spans the sequence -440 to -414 or -413 to -389 of the JAB1 promoter and encompasses the potential C/EBP and GATA sites. (**B**) This labeled probe was incubated with nuclear extract proteins from MCF7 cells. Lane 1 shows binding between probe and nuclear extract. As indicated in lanes 2-4, unlabeled oligonucleotide competitors were included in the binding reaction in 50 and 100-fold molar excess over probe. Lane 4 shows the complexes observed in the presence of probes containing a muation for C/EBP or GATA1.

Task 2 was to evaluate JAB1 genetic changes by fluorescence in situ hybridization (FISH). We have successfully performed FISH on fine needle aspirations (FNAs) from primary breast cancers using probes for choromosome 8 (CEP8) and jab1. To complete this task, we will perform FISH on more FNAs and will further evaluate whether outcome can be determined by JAB1 detection, proving that JAB1 would be a valuable prognostic marker.

Task 3 of this proposal aimed to investigate the role of JAB1 in resistance to Herceptin treatment. We have successfully shown that overexpression of JAB1 in breast cancer cells leads to Herceptin-resistance through inhibition of p27 as projected in Task 3A. To determine whether overexpression of JAB1 provides a protective effect against Herceptin, we used HER2 overexpressing breast cancer cells, SKBR3 and BT474. These cells were transduced with a doxycyclin-regulated (Tet-Off system) adenovirus (Ad-JAB1) and were further treated with Herceptin (10ug/mL) in the absence (-) or presence

(+) of doxycycline (1ug/mL) for 48 h, followed by western blotting and flow cytometry analysis. Herceptin treatment led to an increase in both p27 protein levels and G1 arrest that was inhibited by the overexpression of JAB1 (Fig 3).

We have begun work on Task 3B and successfully shown that inhibition of JAB1 increases the ability of Herceptin to induce G1 arrest. Herceptin resistant cells BT474 C#5 and C#6 were obtained from Timothy Kute. These cells are able to grow similar to the parental cell line, BT474, in the presence of Herceptin. After treatment with Herceptin they have lower phospho-AKT but no increase in nuclear p27. Inhibition of JAB1 by siRNA in these cells increased the ability of Herceptin to induce G1 arrest and inhibit cellular proliferation (Fig 4). Therefore inhibition of JAB1 could be an attractive target for drug development in breast cancer.

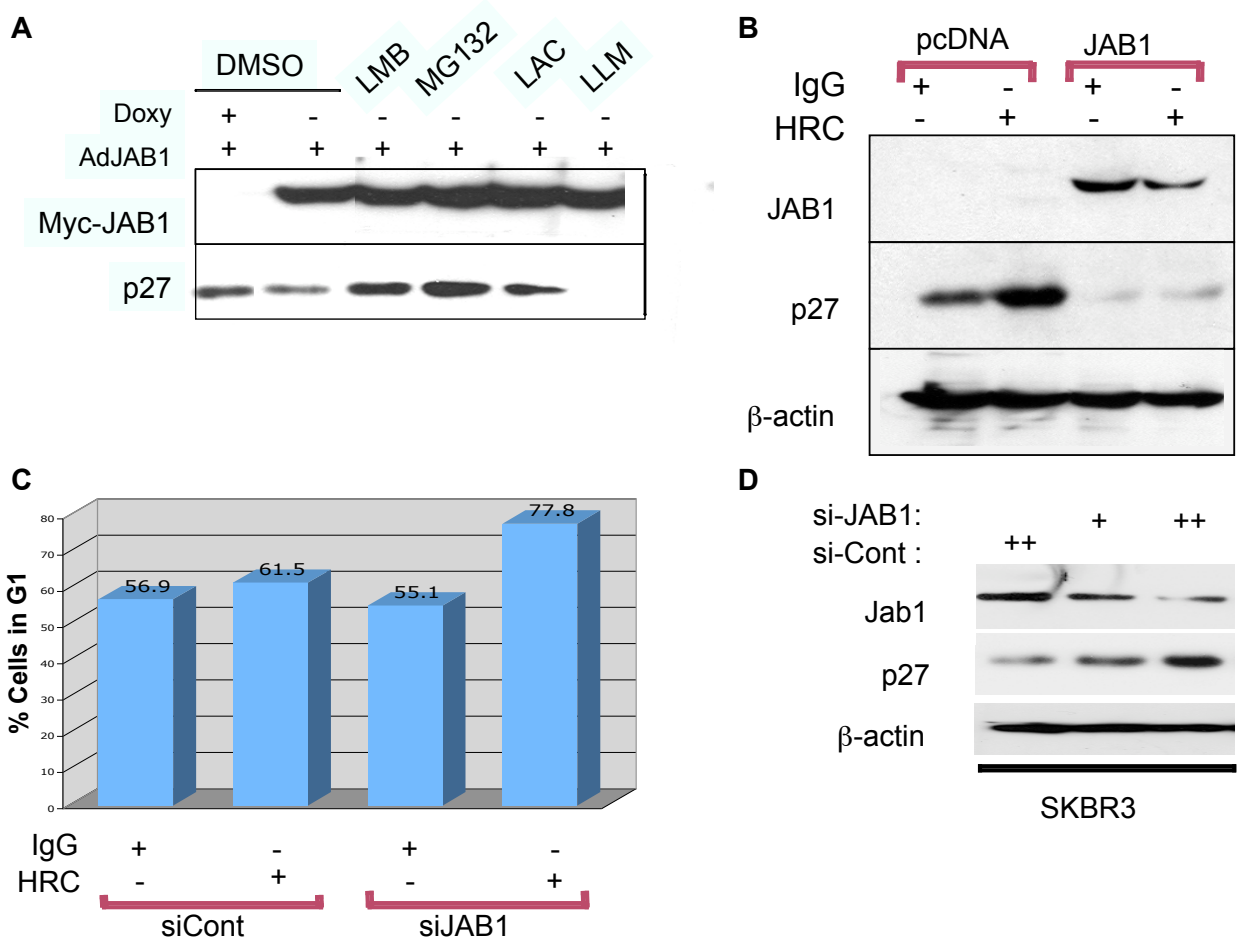


Fig 3. JAB1 overexpression inhibits Herceptin mediated p27 accumulation and depletion of JAB1 by siRNA increases Herceptin induced G1 arrest (**A**), Downregulation of p27 by Ad-Myc-JAB1 is sensitive to 26S proteasome inhibitors (MG132 and LAC) and NES/CRM1/nuclear export inhibitor Leptomycin B (LMB), but not to control inhibitor (LLM). (**B**), JAB1 overexpression inhibits Herceptin mediated p27 accumulation in SKBR3 cells. (**C**), Inhibition of JAB1 by siRNA increases Herceptin mediated G1 arrest in SKBR3 cells by PI staining.(**D**), Knockdown of JAB1 with siRNA enhanced p27 stability

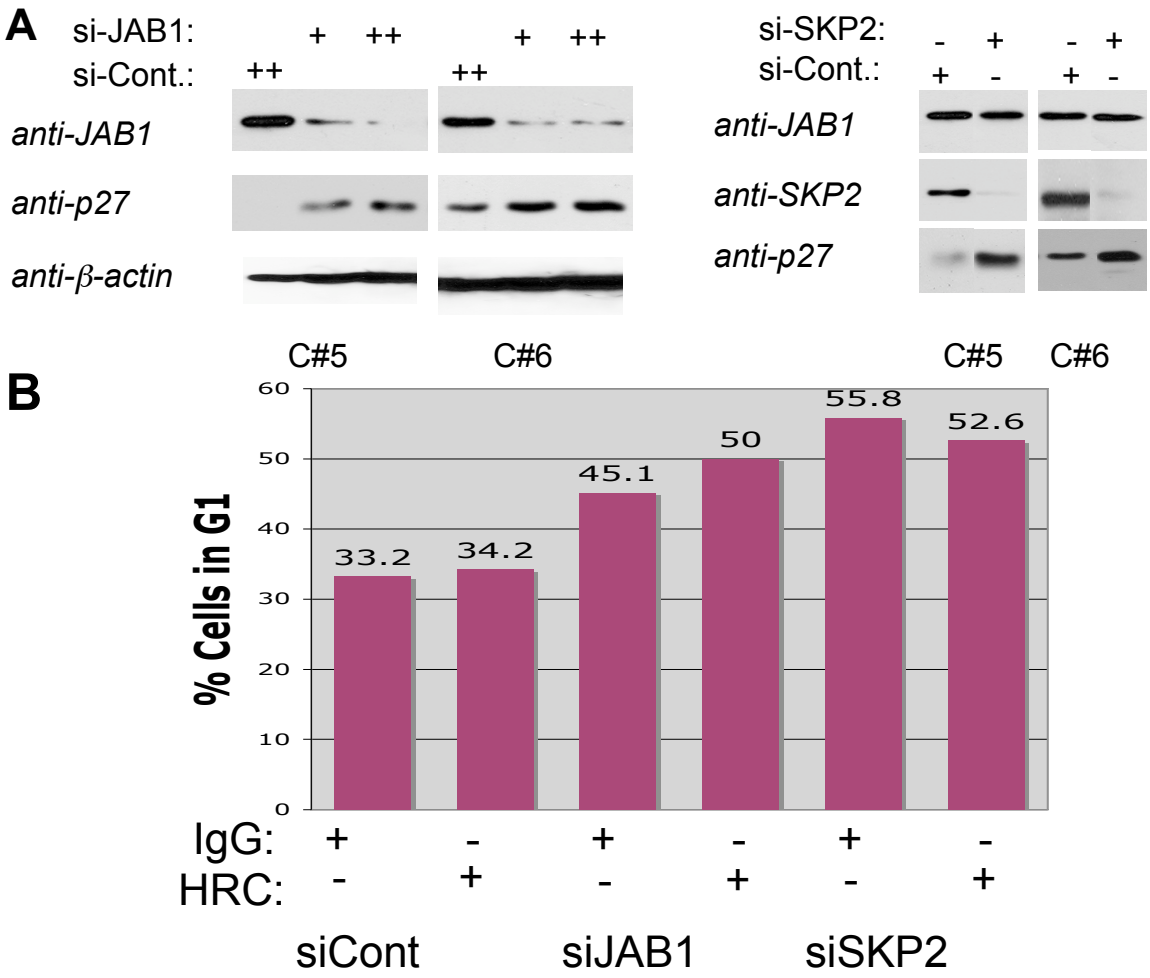


Fig 4. **Silencing JAB1 and SKP2 by siRNA increased sensitivity to Herceptin in Herceptin resistant cells.** (**A**), Inhibition of JAB1 and SKP2 effectively increased p27 levels in C#5 and C#6. (**B**), Inhibition of JAB1 and SKP2 in C#5 resistant cells increased Herceptin induced G1 arrest by PI staining.

Key Research Accomplishments

Task 1A. We have identified the region that is essential for JAB1 transcription

Task 1B. We have identified key transcription factor binding elements in this region, C/EBP and GATA1

Task 1C. We have confirmed binding of these transcription factors to these elements through EMSAs and ChIP.

Task 3A. We have shown that overexpression of JAB1 inhibits Herceptin treatment.

Task 3B. We have demonstrated that inhibition of JAB1 sensitizes breast cancer cells to Herceptin treatment.

Reportable Outcomes

This work has been presented at three conferences and the manuscripts are in preparation.

Conclusions

We have identified two transcription factors that bind the JAB1 promoter leading to an increase in its promoter activity. Further studies will be performed to determine which signaling pathway is responsible for this activation. Also, we have shown that JAB1 overexpression can confer resistance to Herceptin treatment and that inhibition of JAB1 by siRNA sensitizes cells to Herceptin treatment.

Absracts:

Shackleford-Johnson, T.J., Tian, L, Korapati A, Zhang Q, Kute T, Claret, F-X.
Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment.
The 25th Congress of International Association for Breast Cancer Research,
September 15-18, McGill University Cancer Center, Montreal, QC, **Canada**. #SaP38

.Shackleford-Johnson, T.J, Tian L, Korapati A, Zhang Q, Kute T, Claret, F-X.
Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment.
98th American Association for Cancer Research, Annual Meetin,
April 14-18, Los Angeles, CA, **U.S.** #07-AB-4438

Shackleford-Johnson, T.J, Tian L, Korapati A, Zhang Q, Kute T, Claret, F-X.
Role of JAB1 in Resistance to Trastuzumab (Herceptin) Treatment.
Trainee and Recognition Day, MD Anderson Cancer Center
May 25, Houston, TX, **U.S.**

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Shackleford-Johnson, T.J., Tian, L., Rassidakis, G. Z., Claret FX.
Regulation of *Jab*1 gene transcription by Her-2/neu in breast cancer cells.
Journal of Biological Chemistry *In preparation*

Shackleford-Johnson, T.J., Tian, L., Le A-F., Bast, R.C., Kute, T., Claret FX.
Depletion of JAB1 overcomes Trastuzumab-resistance
Cancer Research, *In preparation*